TITLE OF THE INVENTION

MODIFIED CRE RECOMBINASE GENE FOR MAMMALS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is based upon and claims the benefit of priority from the prior Japanese Patent Application No. 11-264364, filed September 17, 1999, the entire contents of which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

The site-specific recombination is a phenomenon found in the process in which λ phage DNA is integrated into a host chromosome. The site specific recombination is mediated by a recombination enzyme called recombinase which catalyzes recombination by recognizing a relatively short specific sequence, whereas the homologous recombination is performed by pairing long homologous nucleic acids. In this respect, the site specific recombination is a biological event completely different from the homologous recombination.

The site-specific recombination can be used to selectively recombinize a gene construct having a desired gene bound thereto, thereby knocking-in or knocking-out the desired gene. Therefore, the site-specific recombination is a very useful technique, especially in the field of embryological engineering for knocking-out or knocking-in a specific gene in a time- or location-controlled manner.

Now, referring to FIG. 1, the mechanism of the site-specific recombination and its application will be explained in brief.

As shown in FIG. 1, unlike the homologous recombination which is initiated with DNA pairing, the site-specific recombination is triggered with binding of recombinase 1 to a specific sequence 3 in DNA 2 to form a DNA-protein complex 5. The recombinase 1 bound to DNA 2 recognizes and binds to a specific sequence 4 which is present in the same DNA 2 or a different DNA and which has the same nucleotide sequence as the specific sequence 3. FIG. 1 shows the case where the specific sequence 3 and 4 are present in the same DNA. The recombinase bound to the specific sequence 3 and 4 catalyses a cleaving/rebinding reaction of single-strand DNA. More specifically, the reaction is performed by two steps: sequentially cleaving the 3' ends of the specific sequence 3 and 4; and binding a cleaved portion of the specific sequence 3 to a site A' and a cleaved portion of the specific sequence 4 to a site A.

As shown in FIG. 1, in the case where the specific sequences are present in the same DNA, the DNA is cleaved into two, one a straight DNA, and the other a cyclic DNA, by the site-specific recombination. The cyclic DNA falls out from the original DNA.

Therefore, if a gene construct having a desired

gene arranged to be fallen off as the cyclic DNA, and a recombinase gene are introduced into a chromosome, and then, the recombinase gene is expressed in a time-controlled and/or location-controlled manner, only the corresponding gene is knocked-out in the time-controlled and/or location-controlled manner.

Alternatively, a gene construct and a recombinase can be introduced into a chromosome to selectively "knock-in" a desired gene in the gene construct. In the gene construct, the desired gene is placed downstream of a first specific sequence and a promoter is placed upstream of a second sequence such that the gene is transferred to be flanked with the promoter after a recombination process in which an intervening sequence between the promoter and the gene is fallen off. Accordingly, knock-in is achieved in time and location controlled manner by expression of the recombinase.

As the recombinase which catalyses the site-specific recombination, FRT recombinase and FLP recombinase which are derived from a yeast, and phage-derived Cre recombinase have been found. However, the yeast-derived FRT and FLP recombines do not work well in mammalian cells.

In contrast, the Cre-loxP system consisting of Cre recombinase and a loxP sequence, which is specifically recognized by Cre recombinase, can be applied to

mammalian cells. Therefore, the Cre-loxP system is used to initiate the site-specific recombination in mammals.

However, since the Cre recombinase is a bacteriophage-derived protein, the codons in the Cre recombinase is not translated efficiently in mammalian cells. Therefore, the Cre recombinase has a drawback in that it is expressed insufficiently.

The present invention is made to overcome the aforementioned drawback associated with the phage-derived Cre recombinase gene. An object of the present invention is to provide a modified Cre recombinase gene for mammals that is expressed in mammalian cells, tissues, organs, or body several times as abundantly as the phage-derived Cre recombinase gene.

BRIEF SUMMARY OF THE INVENTION

To solve the aforementioned object, the present invention provides a modified Cre recombinase gene for mammals (SEQ ID NO: 1) ~~(Sequence number 1)~~.

The present invention is to provide a modified Cre recombinase gene for mammals having a nucleotide sequence represented by (SEQ ID NO: 1) ~~sequence number 1~~.

The modified Cre recombinase gene for mammals of the present invention encodes the same Cre recombinase protein derived from a bacteriophage P1 having an amino acid sequence represented by (SEQ ID NO: 2) ~~sequence number 2~~. However, all codons are modified into those most

frequently used in swine DNA. Therefore, the modified Cre recombinase gene of the present invention is expressed more abundantly in mammals compared to the phage-derived Cre recombinase gene.

Furthermore, the present invention provides a method of knocking-in or knocking-out a desired gene by the modified Cre recombinase gene in a location-controlled and/or time-controlled manner.

Additional objects and advantages of the invention will be set forth in the description which follows, and in part will be obvious from the description, or may be learned by practice of the invention. The objects and advantages of the invention may be realized and obtained by means of the instrumentalities and combinations particularly pointed out hereinafter.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWING

The accompanying drawings, which are incorporated in and constitute a part of the specification, illustrate presently preferred embodiments of the invention, and together with the general description given above and the detailed description of the preferred embodiments given below, serve to explain the principles of the invention.

FIG. 1 is a schematic illustration of a mechanism of site-specific recombination;

FIG. 2 is a schematic illustration of a method of knocking-in a desired gene in a time and/or location

controlled manner by using a modified Cre recombinase gene for mammals of the present invention;

FIG. 3 is a schematic illustration of a method of knocking-out a desired gene in a time and/or location controlled manner by using the modified Cre recombinase gene for mammals of the present invention;

FIGS. 4A-I, 4A-II, 4B-I, 4B-II and 4B-III are Graphs and Western blottings showing the results of Example 2 in which transcription and translation rate of a cDNA of the gene of present invention and that of virus-derived Cre recombinase gene were compared in;

FIG. 5 is a schematic illustration of a gene construct used in Embodiment 2; and

FIG. 6 is a Graphs showing difference in recombination frequency in a mammalian Cre-loxP and a conventional Cre-loxP.

DETAILED DESCRIPTION OF THE INVENTION

More specifically, the codons used herein are as follows (the codons in parentheses are those most frequently used in bacteriophage P1).

Ala:GCC(GCT), Arg:CGC(CGC), Asn:AAC(AAT), Asp:GAC(GAT), Cys:TGC(TGT), Gln:CAG(CAG), Glu:GAG(GAA), Gly:GGC(GGT), His:CAC(CAT), Ile:ATC(ATT), Leu:CTG(CTG), Lys:AAG(AAA), Pro:CCC(CCT), Phe:TTC(TTT), Ser:AGC(TCA), Thr:ACC(ACA), Tyr:TAC(TAT), Val:GTG(GTT)

Note that Met and Trp are not modified since they are encoded only by a single codon.

The codons most frequently used in cDNA of mammals, other than humans such as swine and murines, are the same as the aforementioned codons except Arg. Therefore, the modified Cre recombinase gene for mammals of the present invention can be applied to other mammals. However, if there is a codon whose frequency differs from that of humans, it is preferable that the codon be modified. For example, the codon of Arg, namely, CGG, is preferably modified to CGC in swine and AGA in murines.

The frequency of each of the codons used in cDNA is known with respect to many mammals other than swine and murines. Therefore, the most suitable codon can be selected on the basis of the data of frequency.

No significant difference is observed in frequency in use of each of the codons among mammals. Therefore, the modified Cre recombinase gene for mammals of the present invention can be applied to any mammals even if the codon frequency in a given mammal is unknown. The most frequently used codon for Arg differs between humans, swine and murines. However, since six types of codons for Arg are used with substantially same frequency, even if the codon most frequently used is unknown, no significant problem is posed.

Accordingly, it should be noted that the present invention includes not only a polynucleotide represented by sequence number 1 but also a

polynucleotide obtained by slightly modifying the aforementioned polynucleotide so as to apply it to various mammals other than humans.

Depending upon the expression level required, it is not necessary to replace all codons in a polynucleotide. However, it is generally preferable that all codons should be replaced.

As described, the "modified Cre recombinase gene for mammals" used herein refers to a Cre recombinase gene modified such that it is suitable for use in mammals. The gene is modified so as to having an elevated expression level in mammalian bodies and living tissues, compared to the phage-derived one. Accordingly, use of the gene of the present invention enables to improve efficiency of site-specific recombination in mammalian bodies, organs, tissues, and cells.

More specifically, the expression level of the modified Cre recombinase gene for mammals of the present invention is at least 2-3 times, generally, several times as high as that of the phage-derived one.

The present invention provides a polynucleotide having the modified Cre recombinase gene for mammals to which a regulatory sequence, a marker gene, a nucleotide transport signal, or a Kozak sequence is bound.

The "regulatory sequence" used herein refers to a

nucleic acid sequence which is responsible for an increase/decrease of transcription rate. The regulatory sequence may be, but not limited to, a promoter, enhancer, upstream activation sequence, silencer, upstream suppressor sequence, and attenuator. Each of these regulatory sequences has to be operably linked to the modified Cre recombinase gene for mammals.

The regulatory sequence preferably linked to the modified Cre recombinase gene for mammals is a promoter. More particularly, an inducible promoter is preferred. There are many kinds of inducible promoters that induce gene expression upon interaction with such substances as nutritional elements, hormones, and substrates and the like or by stimulation such as temperature, electromagnetic wave, and oxidative stress and the like. Accordingly, it will be quite easy for one skilled in the art to select an appropriate promoter. Among inducible promoters are included a location-specific promoter and time-specific promoter.

When the inducible promoter is linked to the modified Cre recombinase gene for mammals, it is preferable that the promoter be induced by a substance location-specifically and time-specifically present at the location at which the modified Cre recombinase gene for mammals is to be expressed.

The "marker gene" is a gene indicating that the modified Cre recombinase gene for mammals is introduced

into a target and expressed. The marker gene may be, but not limited to, a drug-resistant gene and a gene encoding a luminescent protein.

The "nucleic acid encoding a nuclear transport signal" refers to a nucleic acid encoding a nuclear transport signal (also called as a nuclear localizing signal) that functions as a signal for transporting a nuclear protein synthesized in a ribosome back into a nucleus. When the expressed Cre recombinase should be localized in the nuclear, the nucleic acid encoding the nuclear transport signal has to be bound to the modified Cre recombinase gene for mammals.

The "Kozak sequence" is a consensus sequence located immediately upstream of a translation initiation site ATG (position -6 to -1). The most frequently appearing sequences from -6 to +4 is GCCRCCATGR (R means G or A). If the Kozak sequence is conserved, it may be possible to increase a translation rate in mammals.

The present invention provides a polynucleotide having a complimentary sequence to the modified Cre recombinase gene for mammals and a polynucleotide to which a regulatory sequence, the marker gene and the like are linked.

Vectors for introducing each of the polynucleotides into individuals, organs, tissues, or cells fall within the scope of the present invention.

The individuals, organs, tissues and cells having the polynucleotide introduced therein also fall within the scope of the present invention. To introduce the polynucleotide into the individuals, organs, tissues or cells, an electroporation, a lipid, and a microinjection (which are well known to one skilled in the art), but not limited to, may be employed.

The modified Cre recombinase gene for mammals can be introduced into any mammalian animals. The modified Cre recombinase gene for mammals may be introduced into, but not limited to, organs including liver, lung, kidney, heart, pancreas, and digestive tracts such as intestine. The modified Cre recombinase gene for mammals may be introduced into, but not limited to, tissues including brain tissue, skin, subcutaneous tissue, epithelium tissue, bone tissue, muscle tissue, and the like. The modified Cre recombinase gene for mammals may be introduced into, but not limited to, cells including all cells constituting the aforementioned organs and tissues, especially, liver cells, pancreatic cells as well as ovary cells, fertilized cells and embryonic stem cells.

The present invention provides a method for knocking-in a desired gene by use of a site-specific recombination reaction which is catalyzed by the Cre recombinase in a location-controlled manner and/or time-controlled manner.

In the method, a first gene construct comprising the modified Cre recombinase gene for mammals and an inducible promoter liked thereto is used to site-specifically recombine a second gene construct comprising two loxP sequences, a desired gene to be knocked-in, and a promoter

As the inducible promoter to be linked in the first gene construct, an inducible promoter can be used that is capable of inducing the expression of the modified Cre recombinase gene for mammals specifically at the site and/or at the time for a desired gene to be knocked-in. With such an inducible promoter the second gene construct will be recombined specifically at a desired site and/or desired time.

The promoter present in the second gene construct is arranged upstream of a first loxP sequence which is present upstream of the other loxP sequence, as shown in FIG. 2. The promoter must be arranged so as to induce the expression of the desired gene to be knocked-in, in other words, so as to render a desired gene functional.

Since the desired gene is placed downstream of the second loxP sequence, an interposed sequence between two loxP sequences is fallen off to make the desired gene linked directly to the first loxP sequence when site-specific recombination is triggered with the specific recognition of loxP sequence by the Cre

recombinase.

A wild-type loxP sequence derived from a bacteriophage P1 has a nucleotide sequence ATAACTTCGTATAGCATACATTATACGAAGTTAT (SEQ ID NO: 3). However, loxP66 sequence (TTCGTATAGCATAGATTATACGAAGTTAT) (SEQ ID NO: 4) and loxP71 sequence (ATAACTTCGTATAGCATACATTATACGAA) (SEQ ID NO: 5) can also be used, in which a deletion is made artificially. Accordingly, the "loxP sequence" used herein may include not only wild one but modified ones which preserve function equivalent to the wild one.

The promoter is linked directly or in close proximity to the first loxP sequence. Therefore, the desired gene which is linked to the first loxP sequence by the site-specific recombination, initiates to be expressed under operation of the promoter.

Therefore, if the first and the second gene constructs are introduced into a desired vital tissue (i.e., organ, tissue or cell taken out from an individual living body) or a desired individual body, the desired gene can be expressed in a location-controlled and/or time-controlled manner.

The first and second gene constructs may be introduced to any vital tissue or individual body. However, it is preferable that they should be introduced into the aforementioned living tissues or mammals which have been enumerated as being suitable recipients for introducing the modified Cre recombinase

gene for mammals.

Transgenic animals to which a desired gene is knocked-in, in a location-controlled and/or time-controlled manner fall within the scope of the present invention. The organs, tissues or cells taken out from the transgenic animals also fall within the scope of the present invention.

Furthermore, the present invention includes the method of knocking-out a desired gene by use of the site-specific recombination in a location-controlled and/or time-controlled manner.

The method of knocking-out a desired gene is attained by the site specific recombination in the same manner as in the method of knocking-in a desired gene. The knocking-out method is basically performed in the same manner as the knocking-in method except that positions of a promoter sequence and a desired gene differ in the second construct.

The knocking-out method and the typical structure of the second construct are schematically shown in FIG. 3.

The knocking-out method is primarily used to terminate the expression of a desired gene. Therefore, it is satisfactory if either the desired gene or the promoter sequence are knocked-out in its entirety or in part, or both of them are knocked-out from the second gene construct by the site-specific recombination.

Therefore, possible arrangements for the loxP sequences, the promoter sequence, and the corresponding gene in the second construct are as follows:

① - promoter - loxP - corresponding gene - loxP -

② - loxP - promoter - corresponding gene - loxP -

③ - loxP - promoter - loxP - corresponding gene -.

As a matter of fact, a single exon to a plurality of exons are generally knocked-out from the desired gene. Therefore, the term "desired gene" usually includes a whole or part of the desired gene.

Therefore, it should be noted that the step of "knocking-out a desired gene" in this text, includes directly knocking out the desired gene itself and knocking out a single to a plurality of exons or the promoter, thereby terminating its expression.

In this case, it is important to select a single to a plurality of exons which can terminate or decrease the activity of the protein to be knocked-out.

In the method of the present invention, the desired gene is generally present between two loxP sequences. Therefore, if the site specific recombination occurs in a location-controlled and/or time-controlled manner, the desired gene is knocked-out from the second gene construct. Hence, it is possible to terminate the expression of a specific gene in a location-controlled and/or time-controlled manner by the method of the present invention.

Any gene can be knocked-out by the method of the present invention. Therefore, the method of the present invention can be widely applied to various fields including the basic medical science and clinical medicine.

The transgenic animals from which a desired gene is knocked-out in a location-controlled and/or time-controlled manner in accordance with the method of the present invention fall within the scope of the present invention. Organs, tissues or cells taken from the transgenic animals also fall within the scope of the present invention. Techniques for preparing the transgenic animals such as transgenic mouse and swine are well known to one skilled in the art.

It is possible to knock-in the first desired gene in a location-controlled and/or time-controlled manner and then knock-out the second desired gene in a location-controlled and/or time-controlled manner, in accordance with the aforementioned two methods. These methods, the transgenic animals created by these methods, organs, tissues, and cells taken from the transgenic animals fall within the scope of the present invention.

As an example, a transgenic swine can be produced for use in organ transplantation by knocking out a xenograft antigen from a specific organ in accordance with the method of the present invention. In the

xenograft transplantation, a severe rejection occurs if the xenograft antigen is present. Therefore, if an animal from which the xenograft antigen is knocked-out, is used, the rejection can be avoided.

However, if the xenograft antigen is knocked-out from a whole body as in a conventional case, various diseases and disorders occur due to the absence of the xenograft antigen.

In contrast, in the method of the present invention, the transgenic swine is produced by knocking out the xenograft antigen from a specific organ, that is, only from a limited organ(s). Therefore, it is possible to prevent diseases or disorders caused by the absence of the antigen.

In the case of swine, it is preferable that a transgenic swine be formed by knocking out α1,3 galactosyl transferase gene since αGal epitope is the biggest xenograft antigen.

Note that the term "xenograft antigen" refers to an antigenic substance present on a xenograft. The antigenic substance causes a rejection in the recipient which receives the xenograft.

The second example is cell transplantation attained by the method of the present invention. A gene construct is prepared by sandwiching a carcinogenic gene derived from a virus such as SV40 between two loxP sequences. Then this gene construct

is introduced into the cell to be transplanted (transplant cell). The resultant cell becomes immortal, so that endless proliferation takes place. When the cells are proliferated to a predetermined level, the Cre recombinase is expressed to remove the carcinogenic gene thereby terminating the proliferation. The proliferation-terminated transplant cell is then transplanted to a recipient.

The transplant cell may be, but not limited to, a liver cell and pancreatic cell.

In a third example, an anti antibody-production-associated-molecule antibody can be knocked-out in a location-controlled and/or time-controlled manner by the method of the present invention.

The term "anti antibody-production-associated-molecule antibody" used herein refers to an antibody against the molecule which directly or indirectly participates in an antibody production mechanism. The anti antibody-production-associated-molecule antibody may be, but not limited to CD3, CD4, CD28, CTLA4, CD80, T cell receptor, major histocompatibility-compatible antigen, cytokines such as IL-4, IL-5, IL-6, cytokine receptor, and the like.

The anti antibody-production-associated-molecule antibody can suppress an immunoreaction associated with transplantation. Therefore, if a virus vector into which the gene of this antibody is integrated, is

introduced into a recipient, the rejection can be drastically suppressed.

However, immuno-suppression is only required in the early stage after the transplantation. If the immune system is suppressed continuously, a significant immunodeficiency will occur. Therefore, if the immune system is suppressed only in the beginning of the transplantation by the method of the present invention, the success rate of organ transplantation can be remarkably increased.

In the foregoing, the method of the present invention has been described in detail with reference to examples, particularly, transplantation. However, these examples are used for only illustrating the present invention. The present invention is not limited by these examples in any sense. One skilled in the art will readily understand that the other examples, such as construction of disease-models (by knocking-in or knocking-out a specific gene in a location-controlled manner or time-controlled manner), gene therapy, the animals and tissues thus obtained are included in the scope of the present invention.

Now, the present invention will be explained more specifically with reference to examples.

Example 1

In this example, a Cre recombinase cDNA construct was synthesized by attaching to a cDNA of a Cre

recombinase gene for mammals the nucleic acid sequence (CCCAAGAAGAAGAGGAAGGTG) (SEQ ID NO:6) encoding a nuclear transport signal: ProLysLysLysArgLysVal. The cDNA used above contains the following codons: Ala:GCC, Arg:CGC, Asn:AAC, Asp:GAC, Cys:TGC, Gln:CAG, Glu:GAG, Gly:GGC, His:CAC, Ile:ATC, Leu:CTG, Lys:AAG, Pro:CCC, Phe:TTC, Ser:AGC, Thr:ACC, Tyr:TAC, Trp:TGG, and Val:GTG. The resultant cDNA construct is compared with a conventional Cre recombinase gene with respect to the level of mRNA and protein.

The cDNA construct was introduced into an expression vector pCAGGS and then transfected into a CHO cell by electroporation. Thereafter, temporary expression was checked and compared. The results are shown in FIGS. 4A-I, 4A-II, 4B-I, 4B-II and 4B-III.

In FIGS. 4A-I, 4B-I and 4B-II, Western blotting is shown in the upper panel and Northern blotting is shown in the lower panel.

As is apparent from the Western blotting, the conventional Cre (wt-Cre) reached a peak on a second day and no expression was observed on a fourth day. Whereas, in the mammalian Cre(s-Cre), the expression level increased until a third day and expression was observed on a fifth day. The amount of the mammalian Cre protein at the third day was about 7 times as large as the conventional case.

According to the Northern blotting, no expression

was observed with respect to mRNA at the third day in both cases. However, mRNA of the mammalian Cre protein (in amount) on a second day and a third day reached 4.2 fold and 6.6 fold as large as the conventional case, respectively.

Note that GAPDH is an index of the amount of mRNA applied onto a gel.

Example 2

In this experiment, frequency of recombination in the presence of the cDNA of the modified Cre recombinase or that of conventional one is checked by use of a gene construct pCXN-YK1 (FIG. 5) containing two loxP sequences and a CAG promoter. The difference in frequency between the two cases was checked.

A gene construct pCXN-YK1 was constructed and transfected in a CHO cell to form a stable cell line (clone 29 and clone 30).

Now, the conventional Cre cDNA and modified Cre cDNA (the amounts are shown in FIG. 6) were introduced respectively in expression vectors pCXN and pMC1. pCXN has a strong promoter activity while pMC1 has a relatively weak promoter activity. The two cDNAs were transfected into clone 29 and clone 30, respectively by electroporation. Thereafter, a frequency of recombination caused by Cre-loxP was evaluated. The expression vector pCXN includes a CAG promoter and an enhancer of cytomegalovirus. The expression vector

pMC1 includes a thymidinekinase promoter and an enhancer of polyoma virus.

As is shown in FIG. 6 (see pMC1-Cre/clone 29 (Panel C) and pMC1-Cre/clone 30 (Panel D)), the modified Cre cDNA shows significantly higher recombination frequency (T study) compared to the conventional one with respect to DNA amounts of 5, 20, and 50 μg.

In the case of pCXN-Cre/clone 29 (panel A, DNA amounts of 5 and 20 μg,), and in the case of pCXN-Cre/clone 30 (Panel B, DNA amount of 20 μg), the modified Cre cDNA shows a significantly high recombination frequency.

From this experiment, it was demonstrated that the modified Cre recombinase gene for mammals shows an extremely higher recombination frequency than the conventional one.

The modified Cre recombinase gene for mammals of the present invention has a notable advantage in that its expression level in bodies, organs, tissues or cells of mammals is several times as high as that of the wild-type virus-derived Cre recombinase gene. Since the expression level of the modified Cre recombinase is high in mammals, the site-specific recombination occurs in mammals with a significantly high frequency.

If the modified Cre recombinase gene for mammals

of the present invention is used, it is possible to knock-in or knock-out a desired gene in a location-controlled and/or time-controlled manner with improved frequency.

If the method of the present invention is used, it is possible to create transgenic animals, organs, tissues or cells into or from which a specific gene is knocked-in or knocked-out in a location-controlled and/or time-controlled manner. The present invention has an immeasurable effect upon clinical medicine and the basic medical science including organ transplantation, gene therapy, and designed animal model for disorder.

Additional advantages and modifications will readily occur to those skilled in the art. Therefore, the invention in its broader aspects is not limited to the specific details and representative experiment shown and described herein. Accordingly, various modifications may be made without departing from the spirit or scope of the general inventive concept as defined by the appended claims and their equivalents.